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(54) Title: LYSOPHOSPHOLIPASE FROM ASPERGILLUS

(57) Abstract: The inventors have isolated lysophospholipases from *Aspergillus* (*A. niger* and *A. oryzae*) having molecular masses of about 68 kDa and amino acid sequences of 600-604 amino acid residues. The novel lysophospholipases have only a limited homology to known amino acid sequences. The inventors also isolated genes encoding the novel enzymes and cloned them into *E coli* strains.

LYSOPHOSPHOLIPASE FROM ASPERGILLUS

FIELD OF THE INVENTION

The present invention relates to lysophospholipases (LPL), methods of using and producing them, as well as nucleic acid sequences encoding them.

BACKGROUND OF THE INVENTION

Lysophospholipases (EC 3.1.1.5) are enzymes that can hydrolyze 2-lysophospholids to release fatty acid. They are known to be useful, e.g., for improving the filterability of an aqueous solution containing a starch hydrolysate, particularly a wheat starch hydrolysate (EP 219,269).

N. Masuda et al., Eur. J. Biochem., 202, 783-787 (1991) describe an LPL from *Penicillium notatum* as a glycoprotein having a molecular mass of 95 kDa and a published amino acid sequence of 603 amino acid residues. WO 98/31790 and EP 808,903 describe LPL from *Aspergillus foetidus* and *Aspergillus niger*, each having a molecular mass of 36 kDa and an amino acid sequence of 270 amino acids.

JP-A 10-155493 describes a phospholipase A1 from *Aspergillus oryzae*. The mature protein has 269 amino acids.

SUMMARY OF THE INVENTION

The inventors have isolated lysophospholipases from *Aspergillus* (*A. niger* and *A. oryzae*) having molecular masses of about 68 kDa and amino acid sequences of 600-604 amino acid residues. The novel lysophospholipases have only a limited homology to known amino acid sequences. The inventors also isolated genes encoding the novel enzymes and cloned them into *E. coli* strains.

Accordingly, the invention provides a lysophospholipase which may be a polypeptide having an amino acid sequence as the mature peptide shown in one of the following or which can be obtained therefrom by substitution, deletion, and/or insertion of one or more amino acids, particularly by deletion of 25-35 amino acids at the C-terminal:

SEQ ID NO: 2 (hereinafter denoted *A. niger* LLPL-1),

SEQ ID NO: 4 (hereinafter denoted *A. niger* LLPL-2),

SEQ ID NO: 6 (hereinafter denoted *A. oryzae* LLPL-1), or

SEQ ID NO: 8 (hereinafter denoted *A. oryzae* LLPL-2).

Further, the lysophospholipase of the invention may be a polypeptide encoded by the lysophospholipase encoding part of the DNA sequence cloned into a

plasmid present in *Escherichia coli* deposit number DSM 13003, DSM 13004, DSM 13082 or DSM 13083.

The lysophospholipase may also be an analogue of the polypeptide defined above which:

i) has at least 70% homology with said polypeptide,

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form,

iii) is an allelic variant of said polypeptide,

Finally, the phospholipase of the invention may be a polypeptide which is encoded by a nucleic acid sequence which hybridizes under high stringency conditions with one of the following sequences or its complementary strand or a subsequence thereof of at least 100 nucleotides:

nucleotides 109-1920 of SEQ ID NO: 1 (encoding *A. niger* LLPL-1),

nucleotides 115-1914 of SEQ ID NO: 3 (encoding *A. niger* LLPL-2),

nucleotides 70-1881 of SEQ ID NO: 5 (encoding *A. oryzae* LLPL-1), or

nucleotides 193-2001 of SEQ ID NO: 7 (encoding *A. oryzae* LLPL-2).

The nucleic acid sequence of the invention may comprise a nucleic acid sequence which encodes any of the lysophospholipases described above, or it may encode a lysophospholipase and comprise:

a) the lysophospholipase encoding part of the DNA sequence cloned into a plasmid present in Escherichia coli DSM 13003, DSM 13004, DSM 13082 or DSM 13083 (encoding *A. niger* LLPL-1, *A. niger* LLPL-2, *A. oryzae* LLPL-1 and *A. oryzae* LLPL-2, respectively),

b) the DNA sequence shown in SEQ ID NO: 1, 3, 5 or 7 (encoding *A. niger* LLPL-1, *A. niger* LLPL-2, *A. oryzae* LLPL-1 and *A. oryzae* LLPL-2, respectively), or

c) an analogue of the DNA sequence defined in a) or b) which

i) has at least 70% homology with said DNA sequence, or

ii) hybridizes at high stringency with said DNA sequence, its complementary strand or a subsequence thereof.

Other aspects of the invention provide a recombinant expression vector comprising the DNA sequence, and a cell transformed with the DNA sequence or the recombinant expression vector.

A comparison with full-length prior-art sequences shows that the mature amino acid sequences of the invention have 60-69 % homology with LPL from *Penicillium notatum* (described above), and the corresponding DNA sequences of the invention show 63-68 % homology with that of *P. notatum* LPL.

A comparison with published partial sequences shows that an expressed sequence tag (EST) from *Aspergillus nidulans* (GenBank AA965865) of 155 amino acid

residues can be aligned with the mature *A. oryzae* LLPL-2 of the invention (604 amino acids) with a homology of 79 %.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

Lysophospholipases of the invention may be derived from strains of *Aspergillus*, particularly strains of *A. niger* and *A. oryzae*, using probes designed on the basis of the DNA sequences in this specification.

Strains of *Escherichia coli* containing genes encoding lysophospholipase were deposited by the inventors under the terms of the Budapest Treaty with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE as follows:

Source organism	Designation of lysophospholipase	Accession number	Date deposited
A. niger	LLPL-1	DSM 13003	18 August 1999
A. niger	LLPL-2	DSM 13004	18 August 1999
A. oryzae	LLPL-1	DSM 13082	8 October 1999
A. oryzae	LLPL-2	DSM 13083	8 October 1999

C-terminal deletion

The lysophospholipase may be derived from the mature peptide shown in SEQ ID NOS: 2, 4, 6 or 8 by deletion at the C-terminal to remove the ω site residue while preserving the lysophospholipase activity. The ω site residue is described in Yoda et al. Biosci. Biotechnol. Biochem. 64, 142-148, 2000, e.g. S577 of SEQ ID NO: 4. Thus, the C-terminal deletion may particularly consist of 25-35 amino acid residues.

A lysophospholipase with a C-terminal deletion may particularly be produced by expression in a strain of *A. oryzae*.

Properties of lysophospholipase

The lysophospholipase of the invention is able to hydrolyze fatty acyl groups in lysophospholipid such as lyso-lecithin (Enzyme Nomenclature EC 3.1.1.5). It may also be able to release fatty acids from intact phospholipid (e.g. lecithin).

Recombinant expression vector

The expression vector of the invention typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a selectable marker, a transcription terminator, a repressor gene or various activator genes. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Production by cultivation of transformant

The lysophospholipase of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the phospholipase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism is preferably a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, such as a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*, e.g. a glucoamylase-producing strain of *A. niger* such as those described in US 3677902 or a mutant thereof. The production of the lysophospholipase in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

Hybridization

The hybridization is used to indicate that a given DNA sequence is analogous to a nucleotide probe corresponding to a DNA sequence of the invention. The hybridization conditions are described in detail below.

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at approx. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55°C, more preferably at least 60°C, more preferably at least 65°C, even more preferably at least 70°C, especially at least 75°C.

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

Alignment and homology

The lysophospholipase and the nucleotide sequence of the invention preferably have homologies to the disclosed sequences of at least 80 %, particularly at least 90 % or at least 95 %, e.g. at least 98 %.

For purposes of the present invention, alignments of sequences and calculation of homology scores were done using a full Smith-Waterman alignment, useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98). Multiple alignments of protein sequences were done using "ClustalW" (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680). Multiple alignment of DNA sequences are done using the protein alignment as a template, replacing the amino acids with the corresponding codon from the DNA sequence.

Lysophospholipase activity (LLU)

Lysophospholipase activity is measured using egg yolk L-α-lysolecithin as the substrate with a NEFA C assay kit.

20 μl of sample is mixed with 100 μl of 20 mM sodium acetate buffer (pH 4.5) and 100 μl of 1% L-α-lysolecithin solution, and incubated at 55°C for 20 min. After 20 min, the reaction mixture is transferred to the tube containing 30 μl of Solution A in NEFA kit preheated at 37°C. After 10 min incubation at 37°C, 600 μl of Solution B in NEFA kit is added to the reaction mixture and incubated at 37°C for 10 min. Activity is measured at 555 nm on a spectrophotometer. One unit of lysophospholipase activity (1 LLU) is defined as the amount of enzyme that can increase the A550 of 0.01 per minute at 55°C.

Use of lysophospholipase

The lysophospholipase of the invention can be used in any application where it is desired to hydrolyze the fatty acyl group(s) of a phospholipid or lysophospholipid, such as lecithin or lyso-lecithin.

As an example, the lysophospholipase of the invention can be used in the preparation of dough, bread and cakes, e.g. to improve the elasticity of the bread or cake. Thus, the lysophospholipase can be used in a process for making bread, comprising adding the lysophospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.) or EP 426211 (Unilever).

The lysophospholipase of the invention can also be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the lysophospholipase. This is particularly applicable to a solution or slurry containing a starch hydrolysate, especially a wheat starch hydrolysate since this tends to be difficult to filter and to give cloudy filtrates. The lysophospholipase may advantageously be used together with a beta-glucanase and/or a xylanase, e.g. as described in EP 219,269 (CPC International).

The lysophospholipase of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the lysophospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil. The process can be conducted according to principles known in the art, e.g. in analogy with US 5,264,367 (Metallgesellschaft, Röhm); K. Dahlke & H. Buchold, INFORM, 6 (12), 1284-91 (1995); H. Buchold, Fat Sci. Technol., 95 (8), 300-304 (1993); JP-A 2-153997 (Showa Sangyo); or EP 654,527 (Metallgesellschaft, Röhm).

EXAMPLES

Materials and methods

Methods

Unless otherwise stated, DNA manipulations and transformations were performed using standard methods of molecular biology as described in Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology",

John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990.

Enzymes

Enzymes for DNA manipulations (*e.g.* restriction endonucleases, ligases etc.) are obtainable from New England Biolabs, Inc. and were used according to the manufacturer's instructions.

Plasmids/vectors

pT7Blue (Invitrogen, Netherlands)
pUC19 (Genbank Accession #: X02514)
pYES 2.0 (Invitrogen, USA).

Microbial strains

E. coli JM109 (TOYOBO, Japan)
E. coli DH12α (GIBCO BRL, Life Technologies, USA)

Aspergillus oryzae strain IFO 4177 is available from Institute for Fermentation, Osaka (IFO) Culture Collection of Microorganisms, 17-85, Juso-honmachi, 2-chome, Yodogawa-ku, Osaka 532-8686, Japan.

A. oryzae BECh-2 is described in Danish patent application PA 1999 01726. It is a mutant of JaL 228 (described in WO 98/12300) which is a mutant of IFO 4177.

Reagents

NEFA test kit (Wako, Japan)
L-α-lysolecithin (Sigma, USA).

Media and reagents

Cove: 342.3 g/L Sucrose, 20 ml/L COVE salt solution, 10mM Acetamide, 30 g/L noble agar.

Cove-2: 30 g/L Sucrose, 20 ml/L COVE salt solution, 10mM, Acetamide, 30 g/L noble agar.

Cove salt solution: per liter 26 g KCl, 26 g MgSO4-7aq, 76 g KH2PO4, 50ml Cove trace metals.

Cove trace metals: per liter 0.04 g NaB4O7-10aq, 0.4 g CuSO4-5aq, 1.2 g FeSO4-7aq, 0.7 g MnSO4-aq, 0.7 g Na2MoO2-2aq, 0.7 g ZnSO4-7aq.

AMG trace metals: per liter 14.3 g ZnSO4-7aq, 2.5 g CuSO4-5aq, 0.5 g NiCl2, 13.8 g FeSO4, 8.5 g MnSO4, 3.0 g citric acid.

YPG: 4 g/L Yeast extract, 1 g/L KH2PO4, 0.5 g/L MgSO4-7aq, 5 g/L Glucose, pH 6.0.

STC: 0.8 M Sorbitol, 25 mM Tris pH 8, 25 mM CaCl2.

STPC: 40 % PEG4000 in STC buffer.

Cove top agarose: 342.3 g/L Sucrose, 20 ml/L COVE salt solution, 10mM Acetamide, 10 g/L low melt agarose.

MS-9: per liter 30 g soybean powder, 20 g glycerol, pH 6.0.

MDU-pH5: per liter 45 g maltose-1aq, 7 g yeast extract, 12 g KH2PO4, 1 g MgSO4-7aq, 2 g K2SO4, 0.5 ml AMG trace metal solution and 25 g 2-morpholinoethanesulfonic acid, pH 5.0.

MLC: 40 g/L Glucose, 50 g/L Soybean powder, 4 g/L Citric acid, pH 5.0.

MU-1: 260 g/L Maltdextrin, 3 g/L MgSO4-7aq, 6 g/L K2SO4, 5 g/L KH2PO4, 0.5 ml/L AMG trace metal solution, 2 g/L Urea, pH 4.5.

Example 1: Cloning and expression of LLPL-1 gene from *A. niger*

Transformation in Aspergillus strain

Aspergillus oryzae strain BECh-2 was inoculated to 100 ml of YPG medium and incubated for16 hrs at 32°C at 120 rpm. Pellets were collected and washed with 0.6 M KCl, and resuspended 20 ml 0.6 M KCl containing a commercial β-glucanase product (Glucanex, product of Novo Nordisk A/S) at the concentration of 30 μl/ml. Cultures were incubated at 32°C at 60 rpm until protoplasts formed, then washed with STC buffer twice. The protoplasts were counted with a hematometer and resuspended in an 8:2:0.1 solution of STC:STPC:DMSO to a final concentration of 2.5x10e7 protoplasts/ml. About 3 μg of DNA was added to 100 μl of protoplasts solution, mixed gently and incubated on ice for 30 min. One ml of SPTC was added and incubated 30 min at 37°C. After the addition of 10 ml of 50°C Cove top agarose, the reaction was poured onto Cove agar plate. Transformation plates were incubated at 32°C for 5 days.

Preparation of a llp1 probe

A strain of Aspergillus niger was used as a genomic DNA supplier.

PCR reactions on *Aspergillus niger* genome DNA was done with the primers HU175 (SEQ ID NO: 9) and HU176 (SEQ ID NO: 10) designed based upon the alignment several lysophospholipases from *Penicillium* and *Neurospora* sp.

Reaction components (1 ng /μl of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ μl in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	Time
1	94°C	2 min
2	92°C	1 min
3	55°C	1 min
4	72°C	1 min
5	72°C	10 min
6	4°C	forever

Steps 2 to 4 were repeated 30 times.

The expected size, 1.0 kb fragment was gel-purified with QIA gel extraction kit (Qiagen, Germany) and ligated into a pT7Blue vector with ligation high (TOYOBO, Japan). The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pHUda94) was sequenced and compared to the *Penicillium* lysophospholipase, showing that a clone encodes the internal part of the lysophospholipase.

Cloning of llpl-1 gene

In order to clone the missing part of the lysophospholipase gene, a genomic restriction map was constructed by using the PCR fragment as probes to a Southern blot of *Aspergillus niger* DNA digested with seven restriction enzymes, separately and probed with 1.0 kb fragment encoding partial lysophospholipase from pHUda94.

A hybridized 4-6 kb SphI fragment was selected for a llpl-1 gene subclone.

For construction of a partial genomic library of *Aspergillus niger*, the genomic DNA was digested with SphI and run on a 0.7 % agarose gel. DNA with a size between 4 to 6 kb was purified and cloned into pUC19 pretreated SphI and BAP (Bacterial alkaline phosphatase). The sphI sub-library was made by transforming the ligated clones into *E. coli* DH12α cells. Colonies were grown on Hybond-N+ membranes (Amersham Pharmacia Biotech, Japan) and hybridized to DIG-labelled (Non-radio isotope) 1.0 kb fragment from pHUda94.

Positive colonies were picked up and their inserts were checked by PCR. Plasmids from selected colonies were prepared and sequenced revealing 5 kb SphI fragment were containing whole llpl-1 gene.

Expression of llpl-1 gene in *Aspergillus oryzae.*

The coding region of the LLPL-1 gene was amplified from genomic DNA of an *Aspergillus niger* strain by PCR with the primers HU188 (SEQ ID NO: 11) and HU189 (SEQ ID NO: 12) which included a EcoRV and a XhoI restriction enzyme site, respectively.

Reaction components (1 ng /µl of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ µl in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	time
1	94°C	2 min
2	92°C	1 min
3	55°C	1 min
4	72°C	2 min
5	72°C	10 min
6	4°C	forever

Steps 2 to 4 were repeated 30 times.

The 2 kb fragment was gel-purified with QIA gel extraction kit and ligated into a pT7Blue vector with Ligation high. The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pLLPL1) was sequenced. The pLLPL1 was confirmed that no changes had happen in the LLPL-1 sequences.

The pLLPL1 was digested with EcoRV and XhoI and ligated into the NruI and XhoI sites in an *Aspergillus* expression cassette (pCaHj483) which has *Aspergillus niger* neutral amylase promoter, *Aspergillus nidulans* TPI leader sequences, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene as a marker. The resultant plasmid was named pHUda103.

The LLPL-1 expression plasmid, pHUda103, was digested with NotI and about 6.1 kb DNA fragment containing *Aspergillus niger* neutral amylase promoter, LLPL-I coding region, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene was gel-purified with QIA gel extraction kit.

The 6.1 kb DNA fragment was transformed into *Aspergillus oryzae* BECh-2. The selected transformants were inoculated in 100 ml of MS-9 media and cultivated at 30°C for 1 day. 3 ml of grown cell in MS-9 medium was inoculated to 100 ml of MDU-pH5 medium and cultivated at 30°C for 3 days. The supernatant was obtained by centrifugation. The cell was opened by mixed with the equal volume of reaction buffer (50 mM KPB-pH 6.0) and glass-beads for 5 min on ice and debris was removed by centrifugation.

The lysophospholipase productivity of selected transformants was determined as the rate of hydrolysis of L-α-lysolecithin at pH 4.5 and 55°C measured in units per ml relative to the activity of the host strain, BECh-2 which is normalized to 1.0. The results shown in the table below clearly demonstrate the absence of in-

creased lysophospholipase activity in supernatants and the presence of increased lysophospholipase activity in cell free extracts.

Strain	Yield (supernatant) Relative activity	Yield (Cell fraction) Relative activity
BECh-2	1.0	1.0
LP3	1.0	4.5
	1.0	4.0
LP8	1.0	6.5
	1.0	5.5

Example 2: Cloning and expression of LLPL-2 gene from *A. niger*

Preparation of a llp2 probe

The same strain of *Aspergillus niger* as in Example 1 was used as a genomic DNA supplier.

PCR reactions on *Aspergillus niger* genomic DNA was done with the primers HU212 (SEQ ID NO: 13) and HU213 (SEQ ID NO: 14) designed based upon amino acid sequences from purified lysophospholipase from AMG 400L (described in Example 4).

Reaction components (1 ng /µl of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ µl in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	Time
1	94°C	2 min
2	92°C	1 min
3	50°C	1 min
4	72°C	1 min
5	72°C	10 min
6	4°C	forever

Steps 2 to 4 were repeated 30 times.

The expected size, 0.6 kb fragment was gel-purified with QIA gel extraction kit (Qiagen, Germany) and ligated into a pT7Blue vector with ligation high (TOYOBO, Japan). The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pHUda114) was sequenced and compared to the *Penicillium* lysophospholipase, showing that a clone encodes the internal part of the lysophospholipase.

Cloning of llpl-2 gene

In order to clone the missing part of the lysophospholipase gene, a genomic restriction map was constructed by using the PCR fragment as probes to a Southern blot of *Aspergillus niger* DNA digested with seven restriction enzymes, separately and probed with 1.0 kb fragment encoding partial lysophospholipase from pHUda114.

A hybridized 4-6 kb XbaI fragment was selected for a llpl-2 gene subclone.

For construction of a partial genomic library of *Aspergillus niger*, the genomic DNA was digested with XbaI and run on a 0.7 % agarose gel. DNA with a size between 4 to 6 kb was purified and cloned into pUC19 pretreated XbaI and BAP (Bacterial alkaline phosphatase). The XbaI sub-library was made by transforming the ligated clones into *E. coli* DH12α cells. Colonies were grown on Hybond-N+ membranes (Amersham Pharmacia Biotech, Japan) and hybridized to DIG-labelled (Non-radio isotope) 1.0 kb fragment from pHUda114.

Positive colonies were picked up and their inserts were checked by PCR. Plasmids from selected colonies were prepared and sequenced revealing 5 kb XbaI fragment were containing whole llpl-2 gene.

Expression of llpl-2 gene in *Aspergillus oryzae*.

The coding region of the LLPL-2 gene was amplified from genomic DNA of an *Aspergillus niger* strain by PCR with the primers HU225 (SEQ ID NO: 15) and HU226 (SEQ ID NO: 16) which included a BglII and a PmeI restriction enzyme site, respectively.

Reaction components (1 ng /μl of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ μl in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	time
1	94°C	2 min
2	92°C	1 min
3	55°C	1 min
4	72°C	2 min
5	72°C	10 min
6	4°C	forever

Step 2 to 4 were repeated 30 times.

The 2 kb fragment was gel-purified with QIA gel extraction kit and ligated into a pT7Blue vector with Ligation high. The ligation mixture was transformed into *E. coli*

JM109. The resultant plasmid (pLLPL2) was sequenced. The pLLPL2 was confirmed that no changes had happen in the LLPL-2 sequences.

The pLLPL2 was digested with BglII and PmeI and ligated into the BamHI and NruI sites in the Aspergillus expression cassette pCaHj483 which has *Aspergillus niger* neutral amylase promoter, *Aspergillus nidulans* TPI leader sequences, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene as a marker. The resultant plasmid was pHUda123.

The LLPL-2 expression plasmid, pHUda123, was digested with NotI and about 6.0 kb DNA fragment containing *Aspergillus niger* neutral amylase promoter, LLPL-2 coding region, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene was gel-purified with QIA gel extraction kit.

The 6.0 kb DNA fragment was transformed into *Aspergillus oryzae* BECh-2. The selected transformants were inoculated in 100 ml of MS-9 media and cultivated at 30°C for 1 day. 3 ml of grown cell in MS-9 medium was inoculated to 100 ml of MDU-pH5 medium and cultivated cultivated at 30°C for 4 days.

The supernatant was obtained by centrifugation. The cell was opened by mixed with the equal volume of reaction buffer (50 mM KPB-pH 6.0) and glass-beads for 5 min on ice and debris was removed by centrifugation.

The lysophospholipase productivity of selected transformants was determined as in Example 1. The results shown in the table below clearly demonstrate the absence of increased lysophospholipase activity in supernatants and the presence of increased lysophospholipase activity in cell free extracts.

Strain	Yield (supernatant) Relative activity	Yield (Cell fraction) Relative activity
BECh-2	1.0	1.0
Fg-9	1.0	22.5
Fg-15	1.0	18.0
Fg-27	1.0	17.0
Fg-33	1.0	14.5

Example 3: Cloning and expression of LLPL genes from *E. coli* clones

Each of the following large molecular weight lysophospholipase (LLPL) genes is cloned from the indicated *E. coli* clone as genomic DNA supplier, and the gene is expressed in *A. oryzae* as described in Examples 1 and 2.

E. coli clone	LLPL
DSM 13003	*A. niger* LLPL-1
DSM 13004	*A. niger* LLPL-2
DSM 13082	*A. oryzae* LLPL-1
DSM 13083	*A. oryzae* LLPL-2

Example 4: Isolation of *A. niger* LLPL-2 from AMG 300L

Purification of LLPL-2 from AMG 300L

A commercially available glucoamylase preparation from *A. niger* (AMG 300L, product of Novo Nordisk A/S) was diluted 10-fold with Milli-Q water and subsequently added ammonium sulfate to 80% saturation. The solution was stirred 1 hour at 4 °C followed by centrifugation on an Sorvall RC-3B centrifuge, equipped with a GSA rotor head (4500 rpm for 35 min). The precipitate was discarded and the supernatant dialysed against 50 mM sodium acetate, pH 5.5. The dialysed solution was applied to a Q-Sepharose (2.6 x 4 cm) column in 50 mM sodium acetate, pH 5.5 at a flow rate of 300 ml h^{-1}. The column was washed (10 x column volume) and proteins were eluted using a linear gradient of 0-0.35 M NaCl in 50 mM sodium acetate, pH 5.5 at a flow rate of 300 ml h^{-1}. Fractions containing activity were pooled, concentrated on an Amicon cell (10 kDa cutoff) to 2.5 ml and applied to Superdex 200 H/R (1.6 x 60 cm) in 0.2 mM sodium acetate, pH 5.5 by draining into the bed. Proteins were eluted isocratically at a flow rate of 30 ml h^{-1}. The purified enzyme showed a specific activity of 86 LLU/mg.

SDS-PAGE analysis showed three protein bands at around 40, 80, and 120 kDa. N-terminal sequencing of the first 23 amino acids revealed that the protein bands at 40 and 120 kDa had identical sequences (shown at the N-terminal of SEQ ID NO: 4), whereas the protein band at 80 kDa was shown to have the sequence shown as SEQ ID NO: 19. IEF analysis showed that LLPL-2 had a pI of around 4.2.

Enzymatic characterisation of LLPL-2

LLPL-2 was show to have a bell-shaped pH-activity profile with optimal activity at pH 4.0. The temperature optimum was found at 50 °C. The enzyme activity was completely stable at pH 4.5 after up to 120 hours incubation at pH 4.5 and 50 °C. LLPL-2 is furthermore completely stable at 50 °C, whereas a half-life of 84 hours was determined at 60 °C. LLPL-2 was not found to be dependent upon addition of mineral salts like sodium or calcium.

Example 5: Identification and sequencing of LLPL-1 and LLPL-2 genes from *A. oryzae*

Cultivation of *A. oryzae*

Aspergillus oryzae strain IFO 4177 was grown in two 20-liter lab fermentors on a 10-liter scale at 34°C using yeast extract and dextrose in the batch medium, and maltose syrup, urea, yeast extract, and trace metals in the feed. Fungal mycelia from the first lab fermentor were harvested by filtering through a cellulose filter (pore size 7-11 microns) after 27 hours, 68.5 hours, 118 hours, and 139 hours of growth. The growth conditions for the second fermentor were identical to the first one, except for a slower growth rate during the first 20 hours of fermentation. Fungal mycelia from the second lab fermentor were harvested as above after 68.3 hours of growth. The harvested mycelia were immediately frozen in liquid N_2 and stored at -80°C.

The *Aspergillus oryzae* strain IFO 4177 was also grown in four 20-liter lab fermentors on a 10-liter scale at 34°C using sucrose in the batch medium, and maltose syrup, ammonia, and yeast extract in the feed. The first of the four fermentations was carried out at pH 4.0. The second of the four fermentations was carried out at pH 7.0 with a constant low agitation rate (550 rpm) to achieve the rapid development of reductive metabolism. The third of the four fermentations was carried out at pH 7.0 under phosphate limited growth by lowering the amount of phosphate and yeast extract added to the batch medium. The fourth of the four fermentations was carried out at pH 7.0 and 39°C. After 75 hours of fermentation the temperature was lowered to 34°C. At 98 hours of fermentation the addition of carbon feed was stopped and the culture was allowed to starve for the last 30 hours of the fermentation. Fungal mycelial samples from the four lab fermentors above were then collected as described above, immediately frozen in liquid N_2, and stored at -80°C.

Aspergillus oryzae strain IFO 4177 was also grown on Whatman filters placed on Cove-N agar plates for two days. The mycelia were collected, immediately frozen in liquid N_2, and stored at -80°C.

Aspergillus oryzae strain IFO 4177 was also grown at 30°C in 150 ml shake flasks containing RS-2 medium (Kofod *et al.*, 1994, *Journal of Biological Chemistry* 269: 29182-29189) or a defined minimal medium. Fungal mycelia were collected after 5 days of growth in the RS-2 medium and 3 and 4 days of growth in the defined minimal medium, immediately frozen in liquid N_2, and stored at -80°C.

Construction of directional cDNA libraries from *Aspergillus oryzae*

Total RNA was prepared by extraction with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion (Chirgwin *et al.*, 1979, *Biochemistry* 18: 5294-5299) using the following modifications. The frozen mycelia were

ground in liquid N_2 to a fine powder with a mortar and a pestle, followed by grinding in a precooled coffee mill, and immediately suspended in 5 volumes of RNA extraction buffer (4 M guanidinium thiocyanate, 0.5% sodium laurylsarcosine, 25 mM sodium citrate pH 7.0, 0.1 M ß-mercaptoethanol). The mixture was stirred for 30 minutes at room temperature and centrifuged (20 minutes at 10 000 rpm, Beckman) to pellet the cell debris. The supernatant was collected, carefully layered onto a 5.7 M CsCl cushion (5.7 M CsCl, 10 mM EDTA, pH 7.5, 0.1% DEPC; autoclaved prior to use) using 26.5 ml supernatant per 12.0 ml of CsCl cushion, and centrifuged to obtain the total RNA (Beckman, SW 28 rotor, 25 000 rpm, room temperature, 24 hours). After centrifugation the supernatant was carefully removed and the bottom of the tube containing the RNA pellet was cut off and rinsed with 70% ethanol. The total RNA pellet was transferred to an Eppendorf tube, suspended in 500 ml of TE, pH 7.6 (if difficult, heat occasionally for 5 minutes at 65°C), phenol extracted, and precipitated with ethanol for 12 hours at -20°C (2.5 volumes of ethanol, 0.1 volume of 3M sodium acetate pH 5.2). The RNA was collected by centrifugation, washed in 70% ethanol, and resuspended in a minimum volume of DEPC. The RNA concentration was determined by measuring $OD_{260/280}$.

The poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose affinity chromatography (Aviv & Leder, 1972, *Proceedings of the National Academy of Sciences USA* 69: 1408-1412). A total of 0.2 g of oligo(dT) cellulose (Boehringer Mannheim, Indianapolis, IN) was preswollen in 10 ml of 1x of column loading buffer (20 mM Tris-Cl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS), loaded onto a DEPC-treated, plugged plastic column (Poly Prep Chromatography Column, BioRad, Hercules, CA), and equilibrated with 20 ml of 1x loading buffer. The total RNA (1-2 mg) was heated at 65°C for 8 minutes, quenched on ice for 5 minutes, and after addition of 1 volume of 2x column loading buffer to the RNA sample loaded onto the column. The eluate was collected and reloaded 2-3 times by heating the sample as above and quenching on ice prior to each loading. The oligo(dT) column was washed with 10 volumes of 1x loading buffer, then with 3 volumes of medium salt buffer (20 mM Tris-Cl, pH 7.6, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS), followed by elution of the poly(A)$^+$ RNA with 3 volumes of elution buffer (10 mM Tris-Cl, pH 7.6, 1 mM EDTA, 0.05% SDS) preheated to 65°C, by collecting 500 µl fractions. The OD_{260} was read for each collected fraction, and the mRNA containing fractions were pooled and ethanol precipitated at -20°C for 12 hours. The poly(A)$^+$ RNA was collected by centrifugation, resuspended in DEPC-DIW and stored in 5-10 mg aliquots at -80°C.

Double-stranded cDNA was synthesized from 5 µg of *Aspergillus oryzae* IFO 4177 poly(A)$^+$ RNA by the RNase H method (Gubler and Hoffman 1983, *supra*; Sambrook *et al.*, 1989, *supra*) using a hair-pin modification. The poly(A)$^+$RNA (5 µg in 5 µl

of DEPC-treated water) was heated at 70°C for 8 minutes in a pre-siliconized, RNase-free Eppendorf tube, quenched on ice, and combined in a final volume of 50 ìl with reverse transcriptase buffer (50 mM Tris-Cl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) containing 1 mM of dATP, dGTP and dTTP, and 0.5 mM of 5-methyl-dCTP, 40 units of human placental ribonuclease inhibitor, 4.81 µg of oligo$(dT)_{18}$-*NotI* primer and 1000 units of SuperScript II RNase H - reverse transcriptase.

First-strand cDNA was synthesized by incubating the reaction mixture at 45°C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gel filtrated through a Pharmacia MicroSpin S-400 HR spin column according to the manufacturer's instructions.

After the gel filtration, the hybrids were diluted in 250 µl of second strand buffer (20 mM Tris-Cl pH 7.4, 90 mM KCl, 4.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.16 mM ßNAD$^+$) containing 200 ìM of each dNTP, 60 units of *E. coli* DNA polymerase I (Pharmacia, Uppsala, Sweden), 5.25 units of RNase H, and 15 units of *E. coli* DNA ligase. Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours, and an additional 15 minutes at 25°C. The reaction was stopped by addition of EDTA to 20 mM final concentration followed by phenol and chloroform extractions.

The double-stranded cDNA was ethanol precipitated at -20°C for 12 hours by addition of 2 volumes of 96% ethanol and 0.2 volume of 10 M ammonium acetate, recovered by centrifugation, washed in 70% ethanol, dried (SpeedVac), and resuspended in 30 ml of Mung bean nuclease buffer (30 mM sodium acetate pH 4.6, 300 mM NaCl, 1 mM $ZnSO_4$, 0.35 mM dithiothreitol, 2% glycerol) containing 25 units of Mung bean nuclease. The single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 minutes, followed by addition of 70 ml of 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction, and ethanol precipitation with 2 volumes of 96% ethanol and 0.1 volume 3 M sodium acetate pH 5.2 on ice for 30 minutes.

The double-stranded cDNAs were recovered by centrifugation (20,000 rpm, 30 minutes), and blunt-ended with T4 DNA polymerase in 30 µl of T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol) containing 0.5 mM of each dNTP, and 5 units of T4 DNA polymerase by incubating the reaction mixture at +16°C for 1 hour. The reaction was stopped by addition of EDTA to 20 mM final concentration, followed by phenol and chloroform extractions and ethanol precipitation for 12 h at -20°C by adding 2 volumes of 96% ethanol and 0.1 volume of 3M sodium acetate pH 5.2.

After the fill-in reaction the cDNAs were recovered by centrifugation as above, washed in 70% ethanol, and the DNA pellet was dried in a SpeedVac. The cDNA pellet was resuspended in 25 µl of ligation buffer (30 mM Tris-Cl, pH 7.8, 10

mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP) containing 2 µg *Eco*RI adaptors (0.2µg/µl, Pharmacia, Uppsala, Sweden) and 20 units of T4 ligase by incubating the reaction mix at 16°C for 12 hours. The reaction was stopped by heating at 65°C for 20 minutes, and then placed on ice for 5 minutes. The adapted cDNA was digested with *Not*I by addition of 20 µl autoclaved water, 5 µl of 10x *Not*I restriction enzyme buffer and 50 units of *Not*I, followed by incubation for 3 hours at 37°C. The reaction was stopped by heating the sample at 65 °C for 15 minutes. The cDNAs were size-fractionated by agarose gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC, Rockland, ME) in 1x TBE (in autoclaved water) to separate unligated adaptors and small cDNAs. The gel was run for 12 hours at 15 V, and the cDNA was size-selected with a cut-off at 0.7 kb by cutting out the lower part of the agarose gel. Then a 1.5% agarose gel was poured in front of the cDNA-containing gel, and the double-stranded cDNAs were concentrated by running the gel backwards until it appeared as a compressed band on the gel. The cDNA-containing gel piece was cut out from the gel and the cDNA was extracted from the gel using the GFX gel band purification kit (Amersham, Arlington Heights, IL) as follows. The trimmed gel slice was weighed in a 2 ml Biopure Eppendorf tube, then 10 ml of Capture Buffer was added for each 10 mg of gel slice, the gel slice was dissolved by incubation at 60°C for 10 minutes, until the agarose was completely solubilized, the sample at the bottom of the tube by brief centrifugation. The melted sample was transferred to the GFX spin column placed in a collection tube, incubated at 25°C for 1 minite, and then spun at full speed in a microcentrifuge for 30 seconds. The flow-through was discarded, and the column was washed with 500 µl of wash buffer, followed by centrifugation at full speed for 30 seconds. The collection tube was discarded, and the column was placed in a 1.5 ml Eppendorf tube, followed by elution of the cDNA by addition of 50 µl of TE pH 7.5 to the center of the column, incubation at 25°C for 1 minute, and finally by centrifugation for 1 minute at maximum speed. The eluted cDNA was stored at -20°C until library construction.

A plasmid DNA preparation for a *Eco*RI-*Not*I insert-containing pYES2.0 cDNA clone, was purified using a QIAGEN Tip-100 according to the manufacturer's instructions (QIAGEN, Valencia, CA. A total of 10 mg of purified plasmid DNA was digested to completion with *Not*I and *Eco*RI in a total volume of 60 ìl by addition of 6 ml of 10x NEBuffer for *Eco*RI (New England Biolabs, Beverly, MA), 40 units of *Not*I, and 20 units of *Eco*RI followed by incubation for 6 hours at 37°C. The reaction was stopped by heating the sample at 65°C for 20 minutes. The digested plasmid DNA was extracted once with phenol-chloroform, then with chloroform, followed by ethanol precipitation for 12 hours at -20°C by adding 2 volumes of 96% ethanol and 0.1 volume of 3 M sodium acetate pH 5.2. The precipitated DNA was resuspended in 25

ml of 1x TE pH 7.5, loaded on a 0.8% SeaKem agarose gel in 1x TBE, and run on the gel for 3 hours at 60 V. The digested vector was cut out from the gel, and the DNA was extracted from the gel using the GFX gel band purification kit (Amersham-Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. After measuring the DNA concentration by $OD_{260/280}$, the eluted vector was stored at -20°C until library construction.

To establish the optimal ligation conditions for the cDNA library, four test ligations were carried out in 10 ìl of ligation buffer (30 mM Tris-Cl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) containing 7 µl of double-stranded cDNA, (corresponding to approximately 1/10 of the total volume in the cDNA sample), 2 units of T4 ligase, and 25 ng, 50 ng and 75 ng of *Eco*RI-*Not*I cleaved pYES2.0 vector, respectively (Invitrogen). The vector background control ligation reaction contained 75 ng of *Eco*RI-*Not*I cleaved pYES.0 vector without cDNA. The ligation reactions were performed by incubation at 16°C for 12 hours, heated at 65°C for 20 minutes, and then 10 µl of autoclaved water was added to each tube. One ìl of the ligation mixtures was electroporated (200 W, 2.5 kV, 25 mF) to 40 µl electrocompetent *E. coli* DH10B cells (Life Technologies, Gaithersburg, MD). After addition of 1 ml SOC to each transformation mix, the cells were grown at 37°C for 1 hour, 50 µl and 5 µl from each electroporation were plated on LB plates supplemented with ampicillin at 100 µg per ml and grown at 37°C for 12 hours. Using the optimal conditions, 18 *Aspergillus oryzae* IFO 4177 cDNA libraries containing 1-2.5x10^7 independent colony forming units was established in *E. coli*, with a vector background of ca. 1%. The cDNA library was stored as (1) individual pools (25,000 c.f.u./pool) in 20% glycerol at -80°C; (2) cell pellets of the same pools at -20°C; (3) Qiagen purified plasmid DNA from individual pools at -20°C (Qiagen Tip 100); and (4) directional, double-stranded cDNA at -20°C.

Aspergillus oryzae EST (expressed sequence tag) Template Preparation

From each cDNA library described, transformant colonies were picked directly from the transformation plates into 96-well microtiter dishes (QIAGEN, GmbH, Hilden Germany) which contained 200 µl TB broth (Life Technologies, Frederick Maryland) with 100 µg ampicillin per ml. The plates were incubated 24 hours with agitation (300 rpm) on a rotary shaker. To prevent spilling and cross-contamination, and to allow sufficient aeration, the plates were covered with a microporous tape sheet AirPore™ (QIAGEN GmbH, Hilden Germany). DNA was isolated from each well using the QIAprep 96 Turbo kit (QIAGEN GmbH, Hilden Germany).

EST Sequencing and Analysis of Nucleotide Sequence Data of the *Aspergillus oryzae* EST Library

Single-pass DNA sequencing of the *Aspergillus oryzae* ESTs was done with a Perkin-Elmer Applied Biosystems Model 377 XL Automatic DNA Sequencer (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA) using dye-terminator chemistry (Giesecke *et al.*, 1992, *Journal of Virology Methods* 38: 47-60) and a pYES specific primer (Invitrogen, Carlsbad, CA). Vector sequence and low quality 3' sequence were removed with the pregap program from the Staden package (MRC, Cambridge, England). The sequences were assembled with TIGR Assembler software (Sutton *et al.*, 1995, *supra*). The assembled sequences were searched with fastx3 (see Pearson and Lipman, 1988, *Proceedings of the National Academy of Science USA* 85: 2444-2448; Pearson, 1990, *Methods in Enzymology* 183: 63-98) against a customized database consisting of protein sequences from SWISSPROT, SWISSPROT-NEW, TREMBL, TREMBLNEW, REMTREMBL, PDB and GeneSeqP. The matrix used was BL50.

Nucleotide sequence analysis

The nucleotide sequence of the lysophospholipase cDNA clones pEST204, and pEST1648 were determined from both strands by the dideoxy chain-termination method (Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467) using 500 ng of Qiagen-purified template (Qiagen, USA), the Taq deoxy-terminal cycle sequencing kit (Perkin-Elmer, USA), fluorescent labeled terminators and 5 pmol of either pYES 2.0 polylinker primers (Invitrogen, USA) or synthetic oligonucleotide primers. Analysis of the sequence data was performed according to Devereux et al., 1984 (Devereux, J., Haeberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387-395).

Example 6: Expression of LLPL-2 in Aspergillus oryzae and Aspergillus niger

Transformation in Aspergillus strain

Aspergillus oryzae strain BECh-2 and an *Aspergillus niger* strain were each inoculated to 100 ml of YPG medium and incubated for16 hrs at 32 °C at 120 rpm. Pellets were collected and washed with 0.6 M KCl, and resuspended 20 ml 0.6 M KCl containing Glucanex at the concentration of 30 µl/ml. Cultures were incubated at 32°C at 60 rpm until protoplasts formed, then washed with STC buffer twice. The protoplasts were counted with a hematometer and resuspended in an 8:2:0.1 solution of STC:STPC:DMSO to a final concentration of 2.5x10e7 protoplasts/ml. About 3 µg of DNA was added to 100 µl of protoplasts solution, mixed gently and incubated on ice for 30 min. One ml of SPTC was added and incubated 30 min at 37 °C. After the ad-

dition of 10 ml of 50 °C Cove top agarose, the reaction was poured onto Cove agar plate. Transformation plates were incubated at 32 °C for 5 days.

Expression of LLPL-2 gene in *Aspergillus niger.*

The coding region of the LLPL-2 gene was amplified from genomic DNA of an *Aspergillus niger* strain by PCR with the primers HU225 (SEQ ID NO: 15) and HU226 (SEQ ID NO: 16) which included a BglII and a PmeI restriction enzyme site, respectively.

Reaction components (1 ng /μl of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ μl in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	time
1	94 °C	2 min
2	92 °C	1 min
3	55 °C	1 min
4	72 °C	2 min
5	72 °C	10 min
6	4 °C	forever

Step 2 to 4 were repeated 30 times.

The 2 kb fragment was gel-purified with QIA gel extraction kit and ligated into a pT7Blue vector with Ligation high. The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pLLPL2) was sequenced, and it was confirmed that no changes had happened in the LLPL-2 sequences.

The pLLPL2 was digested with BglII and PmeI and ligated into the BamHI and NruI sites in the Aspergillus expression cassette pCaHj483 which has *Aspergillus niger* neutral amylase promoter, *Aspergillus nidulans* TPI leader sequences, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene as a marker. The resultant plasmid was named pHUda123.

The LLPL-2 expression plasmid, pHUda123, was transformed into an *Aspergillus niger* strain. Selected transformants were inoculated in 100 ml of MLC media and cultivated at 30 °C for 2 days. 5 ml of grown cell in MLC medium was inoculated to 100 ml of MU-1 medium and cultivated at 30 °C for 7 days.

Supernatant was obtained by centrifugation, and the lysophospholipase activity was measured as described above. The table below shows the lysophospholi-

pase activity from of the selected transformants, relative to the activity of the host strain, MBin114 which was normalized to 1.0.

Strain	Yield (supernatant) Relative activity
MBin114	1.0
123N-33	63
123N-38	150
123N-46	157
123N-48	101

The above results clearly demonstrate the presence of increased lysophospholipase activity in supernatants.

Expression and secretion of C-terminal deleted LLPL-2 gene in *Aspergillus oryzae*

LLPL-2 with the C-terminal deleted (LLPL-2-CD) was made from genomic DNA of a strain of *A. niger* by PCR with the primers HU219 (SEQ ID NO: 17) and HU244 (SEQ ID NO: 18), which included an EagI and a PmeI restriction enzyme site, respectively.

Reaction components (1 ng /ml of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ ml in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	time
1	94 °C	2 min
2	92 °C	1 min
3	55 °C	1 min
4	72 °C	1.5 min
5	72 °C	10 min
6	4 °C	forever

Step 2 to 4 were repeated 30 times.

The 1.3 kb fragment was digested with EagI and PmeI and ligated into the EagI and PmeI sites in the pLLPL-2 having LLPL-2 gene with Ligation

high.(TOYOBO). The ligation mixture was transformed into *E. coli* JM109. The resultant plasmid (pHUda126) was sequenced to confirm that nucleotides 115-1824 of SEQ ID NO: 3 were intact and that nucleotides 1825-1914 of SEQ ID NO: 3 had been deleted, corresponding to a C-terminal deletion of amino acids S571-L600 of LLPL-2 (SEQ ID NO: 4)..

The 2.0 kb fragment encoding LLPL-2-CD was obtained by digesting pHUda126 with BglII and SmaI. The 2.0 kb fragment was gel-purified with the QIA gel extraction kit and ligated into the BamHI and NruI sites in the *Aspergillus* expression cassette pCaHj483 with Ligation high. The ligation mixture was transformed into *E. coli* JM109.

The resultant plasmid (pHUda128) for LLPL-2-CD expression cassette was constructed and transformed into the *A. oryzae* strain, BECh-2. Selected transformants were inoculated in 100 ml of MS-9 media and cultivated at 30 °C for 1 day. 3 ml of grown cell in MS-9 medium was inoculated to 100 ml of MDU-pH5 medium and cultivated cultivated at 30 °C for 3 days.

Supernatant was obtained by centrifugation, and the lysophospholipase activity was measured as described above. The table below shows the lysophospholipase activity from of the selected transformants, relative to the activity of the host strain, BECh-2 which was normalized to 1.0.

Strain	Yield (supernatant) Relative activity
BECh-2	1.0
128-3	9
128-9	7
128-12	33
128-15	11

The above results clearly demonstrate the presence of increased lysophospholipase activity in supernatants.

Example 7: Use of *A. niger* LLPL-2 in Filtration

Filtration performance was determined at 60 °C and pH 4.5 using partially hydrolyzed wheat starch, as follows: The wheat starch hydrolyzate (25 ml in a 100 ml flask) was mixed with LLPL-2 from Example 4 at a dosage of 0.4 L/t dry matter and incubated 6 hours at 60 °C under magnetic stirring. A control was made without enzyme addition. After 6 hours incubation the hydrolyzate was decanted into a glass

and left to settle for 10 min at room temperature. The tendency of the sample to flocculate was determined by visual inspection and ranged as excellent, good, fair, bad, or none. The filtration flux was subsequently determined by running the sample through a filter (Whatman no. 4) and measuring the amount of filtrate after 2, 5, and 10 min. The clarity of the filtered sample was measured spectrophotometrically at 720 nm. The flux of filtrate (ml) was as follows:

Time	Control	LLPL-2
2 min.	4	8
5 min.	8	13
10 min.	12	16

These results indicate that LLPL-2 showed a clear effect on the filtration flux compared to a control sample. Furthermore a clear filtrate was obtained by treatment with LLPL-2.

CLAIMS

1. A lysophospholipase which is:

a) a polypeptide encoded by a lysophospholipase encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* deposit number DSM 13003, DSM 13004, DSM 13082 or DSM 13083, or

b) a polypeptide having an amino acid sequence as the mature peptide shown in SEQ ID NO: 2, 4, 6 or 8, or which can be derived therefrom by substitution, deletion, and/or insertion of one or more amino acids, particularly by deletion of 25-35 amino acids at the C-terminal;

c) an analogue of the polypeptide defined in (a) or (b) which:

i) has at least 70% homology with said polypeptide,

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form, or

iii) is an allelic variant of said polypeptide; or

d) a polypeptide which is encoded by a nucleic acid sequence which hybridizes under high stringency conditions with a complementary strand of the nucleic acid sequence shown as nucleotides 109-1920 of SEQ ID NO: 1, 115-1914 of SEQ ID NO: 3, 70-1881 of SEQ ID NO: 5 or 193-2001 of SEQ ID NO: 7, or a subsequence thereof having at least 100 nucleotides.

2. The lysophospholipase of claim 1 which is native to a strain of *Aspergillus*, preferably *A. niger* or *A. oryzae*.

3. A nucleic acid sequence comprising a nucleic acid sequence which encodes the lysophospholipase of claim 1 or 2.

4. A nucleic acid sequence which comprises:

a) the lysophospholipase encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* DSM 13003, DSM 13004, DSM 13082 or DSM 13083,

b) the nucleic acid sequence shown as nucleotides 109-1920 of SEQ ID NO: 1, 115-1914 of SEQ ID NO: 3, 70-1881 of SEQ ID NO: 5 or 193-2001 of SEQ ID NO: 7,

c) an analogue of the sequence defined in a) or b) which encodes a lysophospholipase and

i) has at least 70% homology with said DNA sequence, or

ii) hybridizes at high stringency with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

5. A nucleic acid construct comprising the nucleic acid sequence of claim 3 or 4 operably linked to one or more control sequences capable of directing the expression of the lysophospholipase in a suitable expression host.

6. A recombinant expression vector comprising the nucleic acid construct of claim 5, a promoter, and transcriptional and translational stop signals.

7. A recombinant host cell transformed with the nucleic acid construct of claim 6.

8. A method for producing a lysophospholipase comprising cultivating the host cell of claim 7 under conditions conducive to production of the lysophospholipase, and recovering the lysophospholipase.

9. The method of the preceding claim wherein the lysophospholipase can be derived from the mature peptide of SEQ ID NO: 2, 4, 6 or 8 or is an analogue thereof, and the host cell is a transformed strain of *A. oryzae*.

10. A process for hydrolyzing fatty acyl groups in a phospholipid or lysophospholipid, comprising treating the phospholipid or lysophospholipid with the lysophospholipase of claim 1 or 2.

11. A process for improving the filterability of an aqueous solution or slurry of carbohydrate origin which contains phospholipid, which process comprises treating the solution or slurry with the lysophospholipase of claim 1 or 2.

12. The process of the preceding claim wherein the solution or slurry contains a starch hydrolysate, particularly a wheat starch hydrolysate.

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tcg atc gcc gtc act cca cgg gcg ttc ccc aat gcc cct gat aaa tat 96
Ser Ile Ala Val Thr Pro Arg Ala Phe Pro Asn Ala Pro Asp Lys Tyr
-5 -1 1 5

gct ccc gca aat gtt tcc tgt ccg tcg act cgt ccc agt atc cgc agt 144
Ala Pro Ala Asn Val Ser Cys Pro Ser Thr Arg Pro Ser Ile Arg Ser
10 15 20 25

gcc gcc gcc ctg tcc acc agt gag aag gat tgg ttg caa gtg cgt cgg 192
Ala Ala Ala Leu Ser Thr Ser Glu Lys Asp Trp Leu Gln Val Arg Arg
30 35 40

aat gag acc ctt gaa ccc atg aag gat ttg ctc ggg cgg ctc aat cta 240
Asn Glu Thr Leu Glu Pro Met Lys Asp Leu Leu Gly Arg Leu Asn Leu
45 50 55

agc tcc ttt gat gcc tcg ggg tac att gac cgt cat aaa aac aat gca 288
Ser Ser Phe Asp Ala Ser Gly Tyr Ile Asp Arg His Lys Asn Asn Ala
60 65 70

tcg aat att cca aac gtg gcc att gcc gtt tca ggt ggt ggt tac cgc 336
Ser Asn Ile Pro Asn Val Ala Ile Ala Val Ser Gly Gly Gly Tyr Arg
75 80 85

gct ttg acc aat ggc gcg ggt gct atc aag gca ttc gat agt cgt acc 384
Ala Leu Thr Asn Gly Ala Gly Ala Ile Lys Ala Phe Asp Ser Arg Thr
90 95 100 105

tcc aac tcc aca gcc cgt gga cag ctc gga ggc ctt ctg cag tcc tct 432
Ser Asn Ser Thr Ala Arg Gly Gln Leu Gly Gly Leu Leu Gln Ser Ser
110 115 120

act tat cta tcg ggc ctc agt ggt ggt gga tgg ctc gtg ggc tcc gtg 480
Thr Tyr Leu Ser Gly Leu Ser Gly Gly Gly Trp Leu Val Gly Ser Val
125 130 135

tac atc aac aac ttc acc act atc ggt gac ctg cag gcc agc gac aag 528
Tyr Ile Asn Asn Phe Thr Thr Ile Gly Asp Leu Gln Ala Ser Asp Lys
140 145 150

gtc tgg gac ttc aag aac tct att ctg gag ggt cct gat gtt aaa cat 576
Val Trp Asp Phe Lys Asn Ser Ile Leu Glu Gly Pro Asp Val Lys His
155 160 165

ttc caa ctg atc aac act gcc gcg tac tgg aag gat ctg tac gat gcg 624
Phe Gln Leu Ile Asn Thr Ala Ala Tyr Trp Lys Asp Leu Tyr Asp Ala
170 175 180 185

gtg aag gat aag aga aac gcc ggg ttc aac act tcg ttg acc gac tac 672
Val Lys Asp Lys Arg Asn Ala Gly Phe Asn Thr Ser Leu Thr Asp Tyr
190 195 200

tgg ggc cgt gct ctc tcc tat cag ttc atc aac gct acc act gat gat 720
Trp Gly Arg Ala Leu Ser Tyr Gln Phe Ile Asn Ala Thr Thr Asp Asp
205 210 215

ggc ggt ccc agt tat acc tgg tcg tcg att gcc ttg ggc gac gat ttc 768
Gly Gly Pro Ser Tyr Thr Trp Ser Ser Ile Ala Leu Gly Asp Asp Phe
220 225 230

aag aag ggc aag atg ccc atg cct atc ctc gtc gcc gat gga cgt aac 816
Lys Lys Gly Lys Met Pro Met Pro Ile Leu Val Ala Asp Gly Arg Asn
235 240 245

ccg ggc gaa ata ctt att gga agt aac tcg act gtg tat gaa ttt aac 864
Pro Gly Glu Ile Leu Ile Gly Ser Asn Ser Thr Val Tyr Glu Phe Asn
250 255 260 265

cca tgg gag ttc ggc tcc ttc gac ccg tca gta tac ggc ttt gca cca 912

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Pro Trp Glu Phe Gly Ser Phe Asp Pro Ser Val Tyr Gly Phe Ala Pro
        270                 275                 280

ttg gag tat ctt gga tcc aat ttc gag aac ggt gaa ctc ccc aag ggg      960
Leu Glu Tyr Leu Gly Ser Asn Phe Glu Asn Gly Glu Leu Pro Lys Gly
    285                 290                 295

gaa tcg tgc gtg cgc ggc ttt gac aat gcg ggt ttt gtc atg ggt acc     1008
Glu Ser Cys Val Arg Gly Phe Asp Asn Ala Gly Phe Val Met Gly Thr
300                 305                 310

agc tct tcc ctg ttt aac cag ttc att ctg cgt ctg aac ggc acc gat     1056
Ser Ser Ser Leu Phe Asn Gln Phe Ile Leu Arg Leu Asn Gly Thr Asp
315                 320                 325

atc cct aat ttc ctc aag gag gcg att gcc gac gtc ttg gaa cat ctg     1104
Ile Pro Asn Phe Leu Lys Glu Ala Ile Ala Asp Val Leu Glu His Leu
330                 335                 340                 345

ggc gaa aac gat gag gac att gca gtt tac gca ccc aac ccc ttc tac     1152
Gly Glu Asn Asp Glu Asp Ile Ala Val Tyr Ala Pro Asn Pro Phe Tyr
                350                 355                 360

aaa tat cgc aat tca acg gca gca tat tcg tca acc cca gag ctg gac     1200
Lys Tyr Arg Asn Ser Thr Ala Ala Tyr Ser Ser Thr Pro Glu Leu Asp
            365                 370                 375

gtg gtc gac gga ggt gaa gat gga cag aac gtg cct cta cac ccg ttg     1248
Val Val Asp Gly Gly Glu Asp Gly Gln Asn Val Pro Leu His Pro Leu
        380                 385                 390

atc cag ccc acc cac aac gtg gat gtg atc ttt gcc gtg gat tcg tcc     1296
Ile Gln Pro Thr His Asn Val Asp Val Ile Phe Ala Val Asp Ser Ser
    395                 400                 405

gct gat acg gac cat agc tgg ccc aac gga tcc tcc ttg atc tac acc     1344
Ala Asp Thr Asp His Ser Trp Pro Asn Gly Ser Ser Leu Ile Tyr Thr
410                 415                 420                 425

tat gaa cgt agc ttg aat act aca ggt atc gcc aac ggg acc tcc ttc     1392
Tyr Glu Arg Ser Leu Asn Thr Thr Gly Ile Ala Asn Gly Thr Ser Phe
                430                 435                 440

cct gcg gtg ccc gac gtc aac acg ttc ctc aac ctt ggc ctg aac aaa     1440
Pro Ala Val Pro Asp Val Asn Thr Phe Leu Asn Leu Gly Leu Asn Lys
            445                 450                 455

cgc ccg acc ttc ttc gga tgc aat tca tcc aac acc agc acc ccg acc     1488
Arg Pro Thr Phe Phe Gly Cys Asn Ser Ser Asn Thr Ser Thr Pro Thr
        460                 465                 470

cca ttg att gtc tac ttg ccc aac gcc cct tac acc gcc gag tcc aac     1536
Pro Leu Ile Val Tyr Leu Pro Asn Ala Pro Tyr Thr Ala Glu Ser Asn
    475                 480                 485

acg tca acc ttc cag ctg gcg tat aag gac caa caa cgc gat gat att     1584
Thr Ser Thr Phe Gln Leu Ala Tyr Lys Asp Gln Gln Arg Asp Asp Ile
490                 495                 500                 505

atc ttg aac ggc tac aac gtc gtc acc cag ggc aat gcc agt gcc gat     1632
Ile Leu Asn Gly Tyr Asn Val Val Thr Gln Gly Asn Ala Ser Ala Asp
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510 515 520

gca aac tgg ccc tcg tgc gtt ggg tgc gct att ctc cag cgg tcc acc 1680
Ala Asn Trp Pro Ser Cys Val Gly Cys Ala Ile Leu Gln Arg Ser Thr
525 530 535

gaa cgt acg aac act aag ctt ccc gat atc tgc aat acc tgc ttc aag 1728
Glu Arg Thr Asn Thr Lys Leu Pro Asp Ile Cys Asn Thr Cys Phe Lys
540 545 550

aat tac tgc tgg gac gga aag acc aac agc acc aca ccg gcc ccc tat 1776
Asn Tyr Cys Trp Asp Gly Lys Thr Asn Ser Thr Thr Pro Ala Pro Tyr
555 560 565

gaa ccg gag cta ttg atg gag gcg tcg act tcc ggg gcc tcg aag gat 1824
Glu Pro Glu Leu Leu Met Glu Ala Ser Thr Ser Gly Ala Ser Lys Asp
570 575 580 585

caa ctg aac cgg aca gct gca gtc atc gcg ttc gca gtt atg ttc ttt 1872
Gln Leu Asn Arg Thr Ala Ala Val Ile Ala Phe Ala Val Met Phe Phe
590 595 600

atg acg atc tag 1884
Met Thr Ile

<210> 6
<211> 627
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<213> Aspergillus oryzae

<400> 6

Met Lys Val Ala Leu Leu Thr Leu Ala Ala Gly Leu Ala Asn Ala Ala
-20 -15 -10

Ser Ile Ala Val Thr Pro Arg Ala Phe Pro Asn Ala Pro Asp Lys Tyr
-5 -1 1 5

Ala Pro Ala Asn Val Ser Cys Pro Ser Thr Arg Pro Ser Ile Arg Ser
10 15 20 25

Ala Ala Ala Leu Ser Thr Ser Glu Lys Asp Trp Leu Gln Val Arg Arg
30 35 40

Asn Glu Thr Leu Glu Pro Met Lys Asp Leu Leu Gly Arg Leu Asn Leu
45 50 55

Ser Ser Phe Asp Ala Ser Gly Tyr Ile Asp Arg His Lys Asn Asn Ala
60 65 70

Ser Asn Ile Pro Asn Val Ala Ile Ala Val Ser Gly Gly Gly Tyr Arg
75 80 85

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Ala Leu Thr Asn Gly Ala Gly Ala Ile Lys Ala Phe Asp Ser Arg Thr
90                  95                  100                 105

Ser Asn Ser Thr Ala Arg Gly Gln Leu Gly Gly Leu Leu Gln Ser Ser
            110                 115                 120

Thr Tyr Leu Ser Gly Leu Ser Gly Gly Gly Trp Leu Val Gly Ser Val
        125                 130                 135

Tyr Ile Asn Asn Phe Thr Thr Ile Gly Asp Leu Gln Ala Ser Asp Lys
    140                 145                 150

Val Trp Asp Phe Lys Asn Ser Ile Leu Glu Gly Pro Asp Val Lys His
    155                 160                 165

Phe Gln Leu Ile Asn Thr Ala Ala Tyr Trp Lys Asp Leu Tyr Asp Ala
170                 175                 180                 185

Val Lys Asp Lys Arg Asn Ala Gly Phe Asn Thr Ser Leu Thr Asp Tyr
            190                 195                 200

Trp Gly Arg Ala Leu Ser Tyr Gln Phe Ile Asn Ala Thr Thr Asp Asp
        205                 210                 215

Gly Gly Pro Ser Tyr Thr Trp Ser Ser Ile Ala Leu Gly Asp Asp Phe
    220                 225                 230

Lys Lys Gly Lys Met Pro Met Pro Ile Leu Val Ala Asp Gly Arg Asn
    235                 240                 245

Pro Gly Glu Ile Leu Ile Gly Ser Asn Ser Thr Val Tyr Glu Phe Asn
250                 255                 260                 265

Pro Trp Glu Phe Gly Ser Phe Asp Pro Ser Val Tyr Gly Phe Ala Pro
            270                 275                 280

Leu Glu Tyr Leu Gly Ser Asn Phe Glu Asn Gly Glu Leu Pro Lys Gly
        285                 290                 295

Glu Ser Cys Val Arg Gly Phe Asp Asn Ala Gly Phe Val Met Gly Thr
    300                 305                 310

Ser Ser Ser Leu Phe Asn Gln Phe Ile Leu Arg Leu Asn Gly Thr Asp
    315                 320                 325
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Ile Pro Asn Phe Leu Lys Glu Ala Ile Ala Asp Val Leu Glu His Leu
330 335 340 345

Gly Glu Asn Asp Glu Asp Ile Ala Val Tyr Ala Pro Asn Pro Phe Tyr
350 355 360

Lys Tyr Arg Asn Ser Thr Ala Ala Tyr Ser Ser Thr Pro Glu Leu Asp
365 370 375

Val Val Asp Gly Gly Glu Asp Gly Gln Asn Val Pro Leu His Pro Leu
380 385 390

Ile Gln Pro Thr His Asn Val Asp Val Ile Phe Ala Val Asp Ser Ser
395 400 405

Ala Asp Thr Asp His Ser Trp Pro Asn Gly Ser Ser Leu Ile Tyr Thr
410 415 420 425

Tyr Glu Arg Ser Leu Asn Thr Thr Gly Ile Ala Asn Gly Thr Ser Phe
430 435 440

Pro Ala Val Pro Asp Val Asn Thr Phe Leu Asn Leu Gly Leu Asn Lys
445 450 455

Arg Pro Thr Phe Phe Gly Cys Asn Ser Ser Asn Thr Ser Thr Pro Thr
460 465 470

Pro Leu Ile Val Tyr Leu Pro Asn Ala Pro Tyr Thr Ala Glu Ser Asn
475 480 485

Thr Ser Thr Phe Gln Leu Ala Tyr Lys Asp Gln Gln Arg Asp Asp Ile
490 495 500 505

Ile Leu Asn Gly Tyr Asn Val Val Thr Gln Gly Asn Ala Ser Ala Asp
510 515 520

Ala Asn Trp Pro Ser Cys Val Gly Cys Ala Ile Leu Gln Arg Ser Thr
525 530 535

Glu Arg Thr Asn Thr Lys Leu Pro Asp Ile Cys Asn Thr Cys Phe Lys
540 545 550

Asn Tyr Cys Trp Asp Gly Lys Thr Asn Ser Thr Thr Pro Ala Pro Tyr
555 560 565

Glu Pro Glu Leu Leu Met Glu Ala Ser Thr Ser Gly Ala Ser Lys Asp

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570                 575                 580                 585

Gln Leu Asn Arg Thr Ala Ala Val Ile Ala Phe Ala Val Met Phe Phe
            590                 595                 600

Met Thr Ile


<210> 7
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cgaccacttc aggtcagt atg aaa ccc aca aca gct gca att gct tta gcc     111
                    Met Lys Pro Thr Thr Ala Ala Ile Ala Leu Ala
                                -35                 -30

ggg ttg ctg tct ggc gtg aca gcg gcc cca ggc cct cat gga gaa agg     159
Gly Leu Leu Ser Gly Val Thr Ala Ala Pro Gly Pro His Gly Glu Arg
    -25                 -20                 -15

att gag agg att gat aga act gtg ttg gaa cgt gca ttg cca aat gct     207
Ile Glu Arg Ile Asp Arg Thr Val Leu Glu Arg Ala Leu Pro Asn Ala
-10                 -5              -1  1               5

ccc gat gga tat gta ccg tcc aac gtc agt tgt cct gcg aat cgc ccg     255
Pro Asp Gly Tyr Val Pro Ser Asn Val Ser Cys Pro Ala Asn Arg Pro
            10                  15                  20

acg gtg cgt agc gca tca tcc ggg ctc tcg agc aat gag acc tcg tgg     303
Thr Val Arg Ser Ala Ser Ser Gly Leu Ser Ser Asn Glu Thr Ser Trp
        25                  30                  35

ttg aaa acc cga cgg gag aag act caa tct gcc atg aaa gat ttc ttc     351
Leu Lys Thr Arg Arg Glu Lys Thr Gln Ser Ala Met Lys Asp Phe Phe
    40                  45                  50

aac cat gtc acg att aag gac ttt gat gct gtc caa tat ctc gac aac     399
Asn His Val Thr Ile Lys Asp Phe Asp Ala Val Gln Tyr Leu Asp Asn
55                  60                  65

cac tcg agt aac acg tcc aat ctt ccc aat att ggt att gcg gtg tct     447
His Ser Ser Asn Thr Ser Asn Leu Pro Asn Ile Gly Ile Ala Val Ser
70                  75                  80                  85

ggt gga ggt tat cgc gcc ctg atg aac ggt gcc gga gcg atc aaa gcg     495
Gly Gly Gly Tyr Arg Ala Leu Met Asn Gly Ala Gly Ala Ile Lys Ala
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          90                   95                  100

ttt gat agc cga acg gag aac tcg acg gcg acg gga cag ttg ggt ggt      543
Phe Asp Ser Arg Thr Glu Asn Ser Thr Ala Thr Gly Gln Leu Gly Gly
        105                 110                 115

ctg cta cag tcg gcg acg tat ctg gct ggt ctg agt ggt ggt gga tgg      591
Leu Leu Gln Ser Ala Thr Tyr Leu Ala Gly Leu Ser Gly Gly Gly Trp
    120                 125                 130

ctg gtg ggg tcg atc tat atc aac aat ttc acc acc att tca gca ctg      639
Leu Val Gly Ser Ile Tyr Ile Asn Asn Phe Thr Thr Ile Ser Ala Leu
  135                 140                 145

cag acc cat gag gat ggt gct gtc tgg cag ttt caa aac tcg att ttt      687
Gln Thr His Glu Asp Gly Ala Val Trp Gln Phe Gln Asn Ser Ile Phe
150                 155                 160                 165

gag ggc cct gac ggc gat agc att cag att ctg gat tct gcg act tac      735
Glu Gly Pro Asp Gly Asp Ser Ile Gln Ile Leu Asp Ser Ala Thr Tyr
            170                 175                 180

tac aag cac gtt tac gat gca gtg caa gac aag aag gat gcg gga tac      783
Tyr Lys His Val Tyr Asp Ala Val Gln Asp Lys Lys Asp Ala Gly Tyr
        185                 190                 195

gaa acc tct atc act gat tat tgg ggt cgc gct ctc tct tat caa tta      831
Glu Thr Ser Ile Thr Asp Tyr Trp Gly Arg Ala Leu Ser Tyr Gln Leu
    200                 205                 210

atc aat gct acc gac ggc ggt ccg agc tat act tgg tcg tcc att gcc      879
Ile Asn Ala Thr Asp Gly Gly Pro Ser Tyr Thr Trp Ser Ser Ile Ala
  215                 220                 225

cta acc gat aca ttt aag cag gca gat atg ccg atg cct ctc ctc gtt      927
Leu Thr Asp Thr Phe Lys Gln Ala Asp Met Pro Met Pro Leu Leu Val
230                 235                 240                 245

gcc gac ggt cgg tat ccc gat gag ctc gtg gtc agc agc aac gct act      975
Ala Asp Gly Arg Tyr Pro Asp Glu Leu Val Val Ser Ser Asn Ala Thr
            250                 255                 260

gtc tat gag ttt aac cct tgg gag ttt ggt act ttt gat cca aca gtc     1023
Val Tyr Glu Phe Asn Pro Trp Glu Phe Gly Thr Phe Asp Pro Thr Val
        265                 270                 275

tac ggg ttt gtg cct cta gaa tac gta ggc tct aaa ttc gac ggt ggt     1071
Tyr Gly Phe Val Pro Leu Glu Tyr Val Gly Ser Lys Phe Asp Gly Gly
    280                 285                 290

tct atc ccc gac aac gag acc tgt gta cgc gga ttc gac aac gcc ggt     1119
Ser Ile Pro Asp Asn Glu Thr Cys Val Arg Gly Phe Asp Asn Ala Gly
  295                 300                 305

ttt gtt atg ggt act tcg tca agt ttg ttc aac cag ttc ttc ctg cag     1167
Phe Val Met Gly Thr Ser Ser Ser Leu Phe Asn Gln Phe Phe Leu Gln
310                 315                 320                 325

gtt aac tca act tcg ctt cct gat ttc ctg aag acg gca ttc tcg gac     1215
Val Asn Ser Thr Ser Leu Pro Asp Phe Leu Lys Thr Ala Phe Ser Asp
            330                 335                 340
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atc ttg gca aag att ggt gaa gaa gat gag gac att gct gtc tat gca    1263
Ile Leu Ala Lys Ile Gly Glu Glu Asp Glu Asp Ile Ala Val Tyr Ala
        345                 350                 355

ccc aac ccg ttc tac aat tgg gcc ccc gtg agc tca cca gca gcc cat    1311
Pro Asn Pro Phe Tyr Asn Trp Ala Pro Val Ser Ser Pro Ala Ala His
    360                 365                 370

caa cag gaa ctc gat atg gtg gac ggt ggc gag gat ctt cag aac att    1359
Gln Gln Glu Leu Asp Met Val Asp Gly Gly Glu Asp Leu Gln Asn Ile
375                 380                 385

cct ctg cat cct tta att cag cca gag cgt cac gta gat gtt atc ttt    1407
Pro Leu His Pro Leu Ile Gln Pro Glu Arg His Val Asp Val Ile Phe
390                 395                 400                 405

gct gtt gac tcc tcc gcc gac acg act tat tct tgg ccc aac ggc aca    1455
Ala Val Asp Ser Ser Ala Asp Thr Thr Tyr Ser Trp Pro Asn Gly Thr
                410                 415                 420

gct ctc gtt gcc act tac gag cgc agc ctg aac tcc acc ggc atc gct    1503
Ala Leu Val Ala Thr Tyr Glu Arg Ser Leu Asn Ser Thr Gly Ile Ala
            425                 430                 435

aac gga acc tca ttc ccc gcg atc cct gac cag aat acc ttt gtt aac    1551
Asn Gly Thr Ser Phe Pro Ala Ile Pro Asp Gln Asn Thr Phe Val Asn
        440                 445                 450

aat ggc ttg aat acg cgg cca acg ttc ttc gga tgt aac agt acg aac    1599
Asn Gly Leu Asn Thr Arg Pro Thr Phe Phe Gly Cys Asn Ser Thr Asn
    455                 460                 465

acc aca ggc cct acg cct ttg gtt gtc tac ctt ccg aac tat cca tac    1647
Thr Thr Gly Pro Thr Pro Leu Val Val Tyr Leu Pro Asn Tyr Pro Tyr
470                 475                 480                 485

gtg tct tac tcg aac tgg tca acc ttc cag cca agc tat gag atc tcc    1695
Val Ser Tyr Ser Asn Trp Ser Thr Phe Gln Pro Ser Tyr Glu Ile Ser
                490                 495                 500

gaa aga gac gac acc atc cgc aac gga tat gat gtg gtg acg atg ggt    1743
Glu Arg Asp Asp Thr Ile Arg Asn Gly Tyr Asp Val Val Thr Met Gly
            505                 510                 515

aac agc act cgt gat ggt aac tgg acg acc tgc gtc ggt tgt gct att    1791
Asn Ser Thr Arg Asp Gly Asn Trp Thr Thr Cys Val Gly Cys Ala Ile
        520                 525                 530

ctg agt cgg tct ttc gag cgc acg aac acc cag gtt ccg gat gcc tgc    1839
Leu Ser Arg Ser Phe Glu Arg Thr Asn Thr Gln Val Pro Asp Ala Cys
    535                 540                 545

acc cag tgc ttc cag aag tac tgc tgg gat ggc act acg aac tcc acc    1887
Thr Gln Cys Phe Gln Lys Tyr Cys Trp Asp Gly Thr Thr Asn Ser Thr
550                 555                 560                 565

aac cct gcc gac tat gag cct gtc acc ctg ttg gag gat agt gct ggt    1935
Asn Pro Ala Asp Tyr Glu Pro Val Thr Leu Leu Glu Asp Ser Ala Gly
                570                 575                 580
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tcc gct ctc tcc ccg gct gtc atc acc acc atc gta gcg acc agt gct 1983
Ser Ala Leu Ser Pro Ala Val Ile Thr Thr Ile Val Ala Thr Ser Ala
585 590 595

gct ctt ttc acc ttg ctg tgagactgga gcaattctgt tggatacggc 2031
Ala Leu Phe Thr Leu Leu
600

tttctttctc ttttctcttc ccaggaacta cttttatata tattgcgata tatcccgact 2091

tttttttttg cttctcttca atttcttcct cctgtgcctt ttagcttgat tgtatttaag 2151

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aaaaaaaaaa aaaaaaaaaa aa 2233

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Met Lys Pro Thr Thr Ala Ala Ile Ala Leu Ala Gly Leu Leu Ser Gly
-35 -30 -25

Val Thr Ala Ala Pro Gly Pro His Gly Glu Arg Ile Glu Arg Ile Asp
-20 -15 -10

Arg Thr Val Leu Glu Arg Ala Leu Pro Asn Ala Pro Asp Gly Tyr Val
-5 -1 1 5 10

Pro Ser Asn Val Ser Cys Pro Ala Asn Arg Pro Thr Val Arg Ser Ala
15 20 25

Ser Ser Gly Leu Ser Ser Asn Glu Thr Ser Trp Leu Lys Thr Arg Arg
30 35 40

Glu Lys Thr Gln Ser Ala Met Lys Asp Phe Phe Asn His Val Thr Ile
45 50 55

Lys Asp Phe Asp Ala Val Gln Tyr Leu Asp Asn His Ser Ser Asn Thr
60 65 70

Ser Asn Leu Pro Asn Ile Gly Ile Ala Val Ser Gly Gly Gly Tyr Arg
75 80 85 90

Ala Leu Met Asn Gly Ala Gly Ala Ile Lys Ala Phe Asp Ser Arg Thr
95 100 105

Glu Asn Ser Thr Ala Thr Gly Gln Leu Gly Gly Leu Leu Gln Ser Ala

110 115 120

Thr Tyr Leu Ala Gly Leu Ser Gly Gly Gly Trp Leu Val Gly Ser Ile
125 130 135

Tyr Ile Asn Asn Phe Thr Thr Ile Ser Ala Leu Gln Thr His Glu Asp
140 145 150

Gly Ala Val Trp Gln Phe Gln Asn Ser Ile Phe Glu Gly Pro Asp Gly
155 160 165 170

Asp Ser Ile Gln Ile Leu Asp Ser Ala Thr Tyr Tyr Lys His Val Tyr
175 180 185

Asp Ala Val Gln Asp Lys Lys Asp Ala Gly Tyr Glu Thr Ser Ile Thr
190 195 200

Asp Tyr Trp Gly Arg Ala Leu Ser Tyr Gln Leu Ile Asn Ala Thr Asp
205 210 215

Gly Gly Pro Ser Tyr Thr Trp Ser Ser Ile Ala Leu Thr Asp Thr Phe
220 225 230

Lys Gln Ala Asp Met Pro Met Pro Leu Leu Val Ala Asp Gly Arg Tyr
235 240 245 250

Pro Asp Glu Leu Val Val Ser Ser Asn Ala Thr Val Tyr Glu Phe Asn
255 260 265

Pro Trp Glu Phe Gly Thr Phe Asp Pro Thr Val Tyr Gly Phe Val Pro
270 275 280

Leu Glu Tyr Val Gly Ser Lys Phe Asp Gly Gly Ser Ile Pro Asp Asn
285 290 295

Glu Thr Cys Val Arg Gly Phe Asp Asn Ala Gly Phe Val Met Gly Thr
300 305 310

Ser Ser Ser Leu Phe Asn Gln Phe Phe Leu Gln Val Asn Ser Thr Ser
315 320 325 330

Leu Pro Asp Phe Leu Lys Thr Ala Phe Ser Asp Ile Leu Ala Lys Ile
335 340 345

Gly Glu Glu Asp Glu Asp Ile Ala Val Tyr Ala Pro Asn Pro Phe Tyr
350 355 360

Asn Trp Ala Pro Val Ser Ser Pro Ala Ala His Gln Gln Glu Leu Asp
365 370 375

Met Val Asp Gly Gly Glu Asp Leu Gln Asn Ile Pro Leu His Pro Leu
380 385 390

Ile Gln Pro Glu Arg His Val Asp Val Ile Phe Ala Val Asp Ser Ser
395 400 405 410

Ala Asp Thr Thr Tyr Ser Trp Pro Asn Gly Thr Ala Leu Val Ala Thr
415 420 425

Tyr Glu Arg Ser Leu Asn Ser Thr Gly Ile Ala Asn Gly Thr Ser Phe
430 435 440

Pro Ala Ile Pro Asp Gln Asn Thr Phe Val Asn Asn Gly Leu Asn Thr
445 450 455

Arg Pro Thr Phe Phe Gly Cys Asn Ser Thr Asn Thr Thr Gly Pro Thr
460 465 470

Pro Leu Val Val Tyr Leu Pro Asn Tyr Pro Tyr Val Ser Tyr Ser Asn
475 480 485 490

Trp Ser Thr Phe Gln Pro Ser Tyr Glu Ile Ser Glu Arg Asp Asp Thr
495 500 505

Ile Arg Asn Gly Tyr Asp Val Val Thr Met Gly Asn Ser Thr Arg Asp
510 515 520

Gly Asn Trp Thr Thr Cys Val Gly Cys Ala Ile Leu Ser Arg Ser Phe
525 530 535

Glu Arg Thr Asn Thr Gln Val Pro Asp Ala Cys Thr Gln Cys Phe Gln
540 545 550

Lys Tyr Cys Trp Asp Gly Thr Thr Asn Ser Thr Asn Pro Ala Asp Tyr
555 560 565 570

Glu Pro Val Thr Leu Leu Glu Asp Ser Ala Gly Ser Ala Leu Ser Pro
575 580 585

Ala Val Ile Thr Thr Ile Val Ala Thr Ser Ala Ala Leu Phe Thr Leu
590 595 600

Leu

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<210> 10
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<212> DNA
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<221> misc_feature
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<210> 11
<211> 30
<212> DNA
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<223> HU188

<400> 11
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<210> 12
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<212> DNA
<213> Artificial/Unknown

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<221> misc_feature
<222> ()..()
<223> HU212

<400> 13
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<210> 14
<211> 21
<212> DNA
<213> Artificial/Unknown

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<222> (19)..()
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1 5 10 15

Gly Lys Lys Asn Ala Ala
20

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 00/00577

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/16 C12N15/63 //(C12N9/16,C12R1:685,C12R1:69)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MASUDA N ET AL: "Primary structure of protein moiety of Penicillium Notatum phospholipase B deduced from the cDNA" EUR J BIOCHEM, vol. 202, 1991, pages 783-787, XP002901491 -& DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; MASUDA N ET AL: "Primary structure of protein moiety of Pencillium Notatum phospholipase B deduced from the cDNA" retrieved from MEDLINE, accession no. 92111525 Database accession no. P39457 XP002901492 62.9% identity in 614 aa overlap abstract --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 2001	08.03.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Yvonne Siösteen

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 31790 A (RÖHM GMBH) 23 July 1998 (1998-07-23) page 3, line 47 ---	1-12
A	US 5 965 422 A (LOEFFLER FRIDOLIN ET AL) 12 October 1999 (1999-10-12) column 27 -column 30 ---	1-12
A	US 6 146 869 A (HARRIS PAUL ET AL) 14 November 2000 (2000-11-14) column 41 -column 42 ---	1-12
A	MUSTRANTA A ET AL: "Comparison of Lipases and Phospholipases in the Hydrolysis of Phospholipids" PROCESS BIOCHEMISTRY, vol. 30, no. 5, 1995, pages 393-401, XP002901493 the whole document -----	1-12

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00577

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claim : all

   relates to lysophospholipases having the amino acid sequence
   SEQ ID No 2
   and related items

2. Claim : all

   relates to lysophospholipases having the amino acid sequece
   SEQ ID No 4
   and related items

3. Claim : all

   relates to lysophospholipases having the amino acid sequence
   SEQ ID No 6
   and related items

4. Claim : all

   relates to lysophospholipases having the amino acid sequece
   SEQ ID No 8
   and related items

INTERNATIONAL SEARCH REPORT

ɔrmation on patent family members

Intern ɲal Application No
PCT/DK 00/00577

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9831790 A	23-07-1998	DE 19701348 A	23-07-1998
		AU 6208098 A	07-08-1998
		BR 9805893 A	24-08-1999
		CA 2243476 A	23-07-1998
		CN 1216061 A	05-05-1999
		CN 1216061 T	05-05-1999
		EP 0904357 A	31-03-1999
		HU 9901640 A	30-08-1999
		US 6140094 A	31-10-2000
US 5965422 A	12-10-1999	DE 19620649 A	27-11-1997
		AU 718990 B	04-05-2000
		AU 1997697 A	27-11-1997
		CA 2205411 A	22-11-1997
		EP 0808903 A	26-11-1997
US 6146869 A	14-11-2000	NONE	